SOP Number	MB-28-00
Title	Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents
Scope	Describes the test methodology, based on the ASTM Standard E2839-11, for producing *C. difficile* spores.
Application	For use in the evaluation of antimicrobial products with *C. difficile* claims.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	1. Pre-reduced medium: Medium free of oxygen. 2. Density gradient medium: HistoDenz™ is a non-ionic gradient medium used to separate spores from vegetative cells and cell fragments on the basis of density. 3. Purified spores: Spore concentration reaches ≥95%. 4. Toxigenic strain: Possesses either toxin A gene (*tcdA*+) or toxin B gene (*tcdB*+) or both.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with chemicals.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-02-06, EQ 05-05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Not applicable
6. Quality Control	1. The organism is a Gram-positive, strictly anaerobic, spore-forming bacterium that produces flat, gray, and irregular colonies on the surface of CABA medium within 48 h at 36±1ºC. 2. Other Quality Control information is documented in the method and on the appropriate form(s) (see section 14).
7. Interferences	1. The test organism must be incubated under strict anaerobic conditions. 2. Harvesting and purification of the spores can be conducted in an aerobic environment. 3. If an anaerobic environment is not maintained, the elevated oxygen will compromise the viability of *C. difficile*.
8. Non-conforming Data	Management of non-conforming data will be specified in the standard test method; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Seal culture plates with Parafilm, or equivalent, to prevent dehydration during the extended anaerobic incubation. 2. Ensure media is pre-reduced prior to use. 3. Inoculated plates and broth should be placed under anaerobic conditions within

	2 hours.
11. Special Apparatus and Materials	1. *Biosafety cabinet*—For maintaining an aseptic work environment. 2. *Sterile centrifuge tubes*—Polypropylene, 15 mL and 50 mL graduated plastic centrifuge tubes with conical bottoms. 3. *Centrifuge with swinging-bucket rotor*—To allow sedimentation of spores for washing and/or concentration. 4. *Micropipette*—Calibrated. 5. *Positive displacement pipette*—To inoculate steel carriers with spores. 6. *Timer*—Any certified timer that can display time in seconds. 7. *Test tubes*—Reusable or disposable 20 × 150 mm for cultures/subcultures. 8. *Inoculating loop*—10 µL transfer loop. 9. *COY Anaerobic chamber*—Supported by a gas mixture consisting of 10 % Hydrogen, 5 % CO_2, and 85 % N_2. 10. *Anaerobic incubator*—Use the incubator at 36 ± 1°C inside the COY anaerobic chamber to support the growth of the organism. 11. *Microscope* with 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option. 12. *Vortex mixer*. 13. *Serological pipettes*—Sterile single-use pipettes of 10.0, 5.0, 1.0 mL capacity. 14. *Cell Scraper*—To gently scrape plates to remove spores for harvesting. 15. *Plate spreader*—To spread inocula on agar to create a uniform lawn. 16. *Microcentrifuge tubes*—Sterile 1.5-mL low-retention (siliconized) microcentrifuge tubes. 17. *Cryovials*—Sterile 2.0 mL cryovials. 18. *Parafilm*™. 19. Media and Reagents a. *Reinforced clostridial medium (RCM)*—For use in rehydrating lyophilized/frozen vegetative culture of test organism. Prepare RCM according to manufacturer's instructions, and pre-reduce in an anaerobic environment for 24 ± 2 h prior to use. b. *RCM plus 15 % glycerol (Cryoprotectant)*—For use as maintenance and cryopreservation medium for vegetative frozen stock (VFS) cultures. Prepare RCM and add 15 % glycerol, autoclave for 20 min at 121°C, and pre-reduce in an anaerobic environment for 24 ± 2 h

	prior to use. c. *CDC anaerobic 5 % sheep blood agar (CABA)* —Commercially available pre-reduced, used for sporulation. d. *Brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT)* —Pre-reduced recovery media for enumeration of viable spores. e. *Phosphate-buffered saline (PBS)*—Prepare 10X stock solution of PBS by dissolving 492 g PBS powder in 5 L of deionized water. Dilute 1:10 (1 part 10X solution plus 9 parts deionized water) to obtain 1X solution, distribute into bottles and autoclave for 20 min at 121°C. f. *Phosphate-buffered saline (PBS) containing 0.1 % Tween 80 (ST80)*—Washing reagent; add 2.0 mL of polysorbate 80 (Tween 80, or equivalent) to 2.0 L of PBS (1X) solution in a 2 L volumetric flask and bring solution to volume with PBS. Distribute into bottles and autoclave for 20 min at 121ºC. g. *Water*—Use sterile deionized water as diluent. h. *Certified Hydrochloric acid*—Use 2.5 M HCl for quantitative acid resistance test. i. *HistoDenz*™—Prepare a 50 % (w/v) solution in deionized water. This is a density gradient medium for spore purification. Pass the solution through a sterile 0.45 µm filter to sterilize. 20. Test Organism: a. *Clostridium difficile* (ATCC 43598), a toxigenic strain (*tcdA*-, *tcdB*+), obtained from ATCC or another reputable vendor. The strain produces Toxin B only (presence of *tcdB* gene by PCR).
12. Procedure and Analysis	
12.1 Preparation of Frozen Stock Cultures of Test Organism	a. *Clostridium difficile received in lyophilized vegetative form*: To reinitiate a new stock culture, reconstitute contents of the lyophilized culture with 0.5 mL of sterile pre-reduced RCM in the COY anaerobic chamber as per the manufacturer's instructions. After rehydration, aseptically transfer the vial contents to a tube containing 4 ± 1 mL of pre-reduced RCM, and mix by gentle vortexing. b. *Clostridium difficile received as frozen vegetative culture*: To reinitiate a new stock culture, thaw frozen culture at room temperature. Transfer the contents to a tube containing 4 ± 1 mL of

	sterile pre-reduced RCM in the COY anaerobic chamber, and mix by gentle vortexing. c. Spread plate 100 µL of the reconstituted culture on five CABA plates. Also streak one CABA plate for isolation to check for culture purity. Invert plates and incubate anaerobically at 36±1°C for 48±4 h. d. Following incubation, add 2 mL of cryoprotectant to each CABA plate. e. Using a sterile cell scraper, gently scrape culture from the surface of the plate, aspirate with a pipette and transfer to a 15-mL conical tube. f. Repeat this process for the remaining plates. Pool the cryoprotectant suspensions, mix thoroughly, and pipette 1 to 1.5 mL aliquots into cryovials; cap tightly. g. Store the cryovials at –80±5°C. These tubes are the Frozen Stock Culture (FSC). h. After a minimum of one week of freezing, thaw a FSC cryovial at room temperature inside the COY chamber. i. Vortex suspension thoroughly, and dilute 1 mL in a 1:10 series out to 10^{-6} in ST80. Spread-plate 100 µL of diluted suspension on BHIY-HT in duplicate. Invert plates and incubate anaerobically at 36±1°C for 48±4 h. j. Record the number of CFU/plate to determine the CFU/mL. The titer should be >8 log/mL to ensure that the FSC contains a sufficiently high titer. k. Prior to preparation of a spore preparation (See 12.2), streak three CABA plates with the FSC. Incubate two plates anaerobically, and the third one aerobically at 36±1°C. Do not use the culture if there is any growth on the plate incubated aerobically. Inspect plates incubated anaerobically for purity and colony characteristics typical of *C. difficile*.
12.2 Preparation of a spore suspension from FSC	a. Using one of the two plates from 12.1 k, inoculate 10 mL of pre-reduced RCM with an isolated colony and mix well by vortexing. Incubate anaerobically at 36±1°C for 24±2 h. b. After incubation, inoculate each of a minimum of ten CABA plates with 100 µL of the RCM broth culture. Spread the inoculum evenly using a disposable sterile spreader to create a lawn. c. Seal culture plates with Parafilm, or equivalent, to prevent dehydration during incubation. Invert plates and incubate

	anaerobically for 7 to 10 days at 36±l°C and ≥70% relative humidity. d. Open one or two plates after 24±2 h of incubation to inspect for confluent growth. Reseal the plate and continue incubation. If growth is not confluent, inspect the remaining plates and discard. e. Over the 7-10 day incubation time frame, periodically prepare wet-mount samples of *C. difficile* from a sample plate and inspect under phase-contrast microscopy. Spores appear bright and ovular, while vegetative cells appear dark and rod-shaped. f. Note degree of conversion of vegetative cells to spores and estimate the approximate ratio of spores to vegetative cells to determine the optimal time for harvesting. g. When the percent of spores reaches ≥ 90% (Attachment 1), discontinue incubation and place the CABA plates inside a BSC. h. Harvest growth from each plate by adding 5 mL of ST80 to each plate, and gently scraping the surface of the plate with a cell scraper or spreader to dislodge the spores. Do not break the surface of the agar. i. Using a 10 mL sterile serological pipette, aspirate as much of the microbial suspension as possible from each plate, and pool it in sterile 50 mL plastic conical tubes. j. Centrifuge tubes at 4500×g for 15 min. k. Discard the supernatant and resuspend the pellet in 20 to 30 mL of ST80. Cap the tubes tightly and disaggregate the pellet by vortexing. This step is the first wash. l. Repeat the washing step two more times. Note that resuspended contents collected from two or more tubes can be combined in one tube after pellets have been disaggregated. Mix by vortexing. m. After the third wash, discard the supernatant and resuspend the pellet in 4 mL of ST80. Mix well by vortexing to disaggregate the pellet. Heat the spore suspension in a heat block for l0±1 min at 65±2°C. n. To ensure that the spore suspension has reached 65±2°C, place a thermometer in an identical tube containing the same volume of deionized water alongside the spore suspension and start the timer once the temperature of the water has reached 65±2°C. o. After heat treatment allow the suspension to cool to room temperature. p. Prepare a wet-mount of the well-vortexed, heat-treated spore suspension and observe at least five fields using a phase-contrast microscope. The percent of spores to vegetative cells should be

	approximately 90%. q. Perform serial 10-fold dilutions of the spore suspension out to 10^{-6} in ST80. r. Spread-plate 0.1 mL of the appropriate dilutions on BHIY-HT in duplicate. s. Once the inocula have dried, invert plates and incubate anaerobically at 36±1°C for 48±4 h. Record the numbers of CFU. The titer should be $>10^8$ viable spores/mL.
12.3 Spore Purification	a. Make a 50% (w/v) solution of HistoDenz in sterile deionized water and pipet 5 mL into each of four sterile 15 mL plastic conical tubes. Layer 1 mL of spore suspension on top of the HistoDenz in each of the four 15 mL tubes. b. Centrifuge tubes at 4,500×g for 10 min using a swinging bucket rotor (see Note 1). NOTE 1: Use of a swinging bucket rotor is essential for proper layer removal and spore retention. c. Carefully remove, using a 1 mL pipet, the top three layers – an upper clear layer, a dense second layer, and a clear third layer – (Attachment 3) and discard, leaving the pellet and the 3 to 4 mm cloudy layer above the pellet undisturbed. d. Use a pipette to resuspend the pellet, vortex and transfer 1 mL aliquots to siliconized micro- centrifuge tubes until the entire volume has been transferred. e. Centrifuge the microcentrifuge tubes at 16,000×g for 5 min. Discard the supernatant and resuspend the pellet in 1 to 1.5 mL of cold (2 to 5°C) ST-80. f. Cap the tubes and mix by vortexing to thoroughly disaggregate the pellet. g. Centrifuge the microcentrifuge tubes at 16,000×g for 2 min. Discard the supernatant and resuspend the pellet in 1 to 1.5 mL of cold (2 to 5°C) ST80. Cap the tubes and mix by vortexing to thoroughly disaggregate the pellet. This step is the first wash. h. Repeat (h) procedure two additional times, for a total of three washes. i. Discard the supernatant and resuspend the pellet in each microcentrifuge tube with 0.5 mL of sterile ST80 and pool. This is the final purified spore suspension (Attachment 2). j. Determine spore purity using procedures stated in microscopic

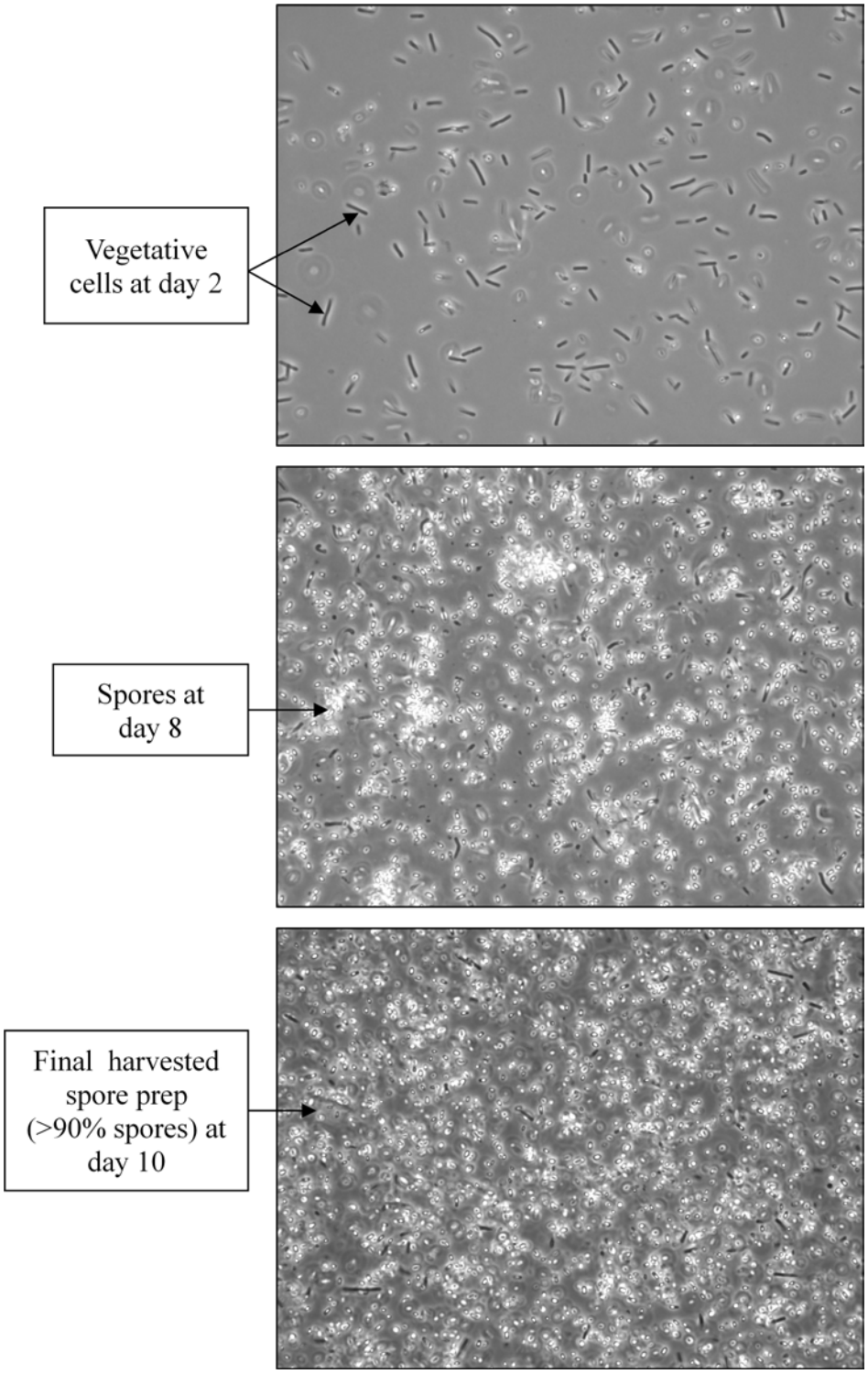
		evaluation of spore suspension. Calculate purity of the spore suspension using the formula presented below in 13.1. The purity of spores should be ≥95%. k. Perform procedures specified in evaluation of titer and calculate the titer of the purified spore suspension using the formula presented in 13.2. The titer of spores should be >8 $\log_{10}$/mL.
12.4	Quantitative Hydrochloric Acid (HCl) Resistance Test	a. Place 990 µL of 2.5 M HCl into one 1.5 mL low-retention (siliconized) microcentrifuge tube; for the control, place 990 µL of ST80 into one 1.5 mL low-retention (siliconized) microcentrifuge tube. b. Using a positive displacement pipette, transfer10 µL of purified spore suspension (spore titer of >8 $\log_{10}$/mL) into each microcentrifuge tube to result in a suspension containing >10^6 spores/mL. Vortex each tube. c. Incubate the acid/spore suspensions and the control tubes for 10 min ±30 sec at room temperature. d. At the end of each incubation period, transfer 0.1 mL from the acid/spore tube and the control tube to tubes containing 900 µL of ST80 to dilute/neutralize the acid. e. Serially dilute the neutralized suspensions out to 10^{-6} in ST80 and spread-plate 0.1 mL aliquots from appropriate dilutions, in duplicate, on BHIY-HT. Invert plates and incubate for 48±4 h at 36±1°C under anaerobic conditions. f. The spores are considered acid-resistant if the log reduction is between 0 to 2 following 10 min of exposure, as compared with the control. g. Determine the $\log_{10}$ reduction following the HCl treatment using the formula presented in 13.3. NOTE: Once the spore suspension fulfills all the required criteria (e.g., titer, spore purity and acid resistance), make aliquots, label appropriately and store at -20ºC up to six months.

13. Data Analysis/ Calculations	1. Determine spore suspension purity by the following calculation: Where A = mean spore count, and B = mean vegetative cell count. 2. Determine the titer of the spores in suspension using the following calculation: Where A = mean colony count at dilution plated, B = reciprocal of dilution used, and C = volume plated. 3. Determine the $\log_{10}$ reduction following HCl treatment: *Log_{10} Reduction (LR) = LC-LH* Where LC= Log_{10} of viable spores after control treatment, and LH= Log_{10} of viable spores after HCl treatment.
14. Forms and Data Sheets	1. Attachment 1: Monitoring percent sporulation of *C. difficile*. 2. Attachment 2: Purified *C. difficile* spores, using a density gradient medium (HistoDenz), depicting ≥99% purity. 3. Attachment 3: Separation of vegetative cells (A) and cell fragments (B) from *C. difficile* spores upon purification with HistoDenz. 4. Test Sheets: Test sheets are stored separately from the SOP under the following file names: *C. difficile* Spore Titer Form — MB-28-00_F1.docx HCl Resistance Test Form — MB-28-00_F2.docx HCl Resistance Test Dilution and Results Form — MB-28-00_F3.docx
15. References	1. ASTM E2839-11, Standard Test Method for Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents. ASTM International 2011. 2. EPA Guidance for the Efficacy Evaluation of Products with Sporicidal Claims against *Clostridium difficile*, http://www.epa.gov/oppad001/ cdif-guidance.html, 2009. 3. Hasan, J. A., Japal, K. M., Christensen, E. R., & Samalot-Freire, L. C., "Development of methodology to generate *Clostridium difficile* spores for use in the efficacy evaluation of disinfectants, a precollaborative investigation," *J. AOAC Int*, Vol 94, 2011, pp. 259–272. 4. Standard Methods for the Examination of Water and Wastewater,

	American Public Health Association, Washington, D.C, 2012. 5. Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Ed., Centers for Disease Control and Prevention, and National Institute of Health, Washington DC, 2009.

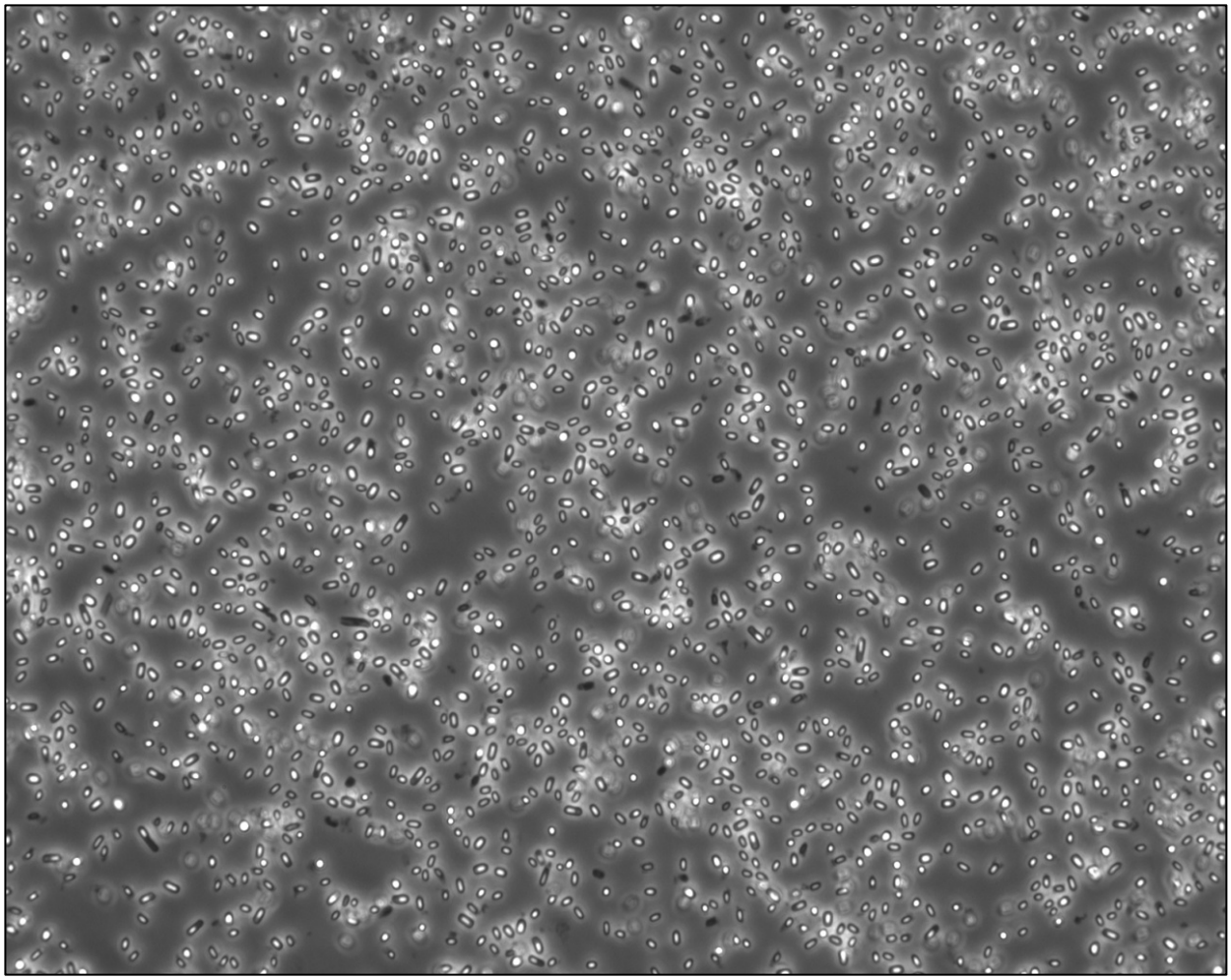
Attachment 1

Monitoring percent sporulation of *C. difficile* (ATCC 43598) during incubation at 36 ± 1ºC under phase contrast microscopy (magnification 1000X)



Attachment 2

Purified *C. difficile* spores (ATCC 43598), using HistoDenz, depicting ≥99% purity



Attachment 3

Separation of vegetative cells (A) and cell fragments (B) from *C. difficile* spores (ATCC 43598) upon purification with HistoDenz.